PROJECT PLAN PROSPECTUS
NP 106 Aquaculture
Panel Review
June-August 2004

Old CRIS Project Number

6435-43440-040-00D

Research Management Unit

6435-56 Food Processing and Sensory Quality

Location

New Orleans, LA

Title

Mitigation of Off-flavors in Catfish Aquaculture

Investigators

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Scientific Staff Years

2.2

Planned Duration

60 months

Signatures

/s/ Elaine T. Champagne	02/03/04
Elaine T. Champagne, Research Leader	Date approved
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John Patrick Jordan, Center Director	Date approved
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Key Words

aquaculture, catfish, off-flavor, cyanobacteria, algae, geosmin, 2-methylisoborneol

Objectives

Objective 1: Identify, characterize and determine the killing efficiency of viruses specific for off-flavor-producing cyanobacteria in aquaculture systems.

Objective 2: Identify, characterize and develop novel bacterial strains that metabolize (biodegrade) geosmin and 2-methylisoborneol and develop them for use in eliminating those chemicals from aquaculture systems.

Objective 3: Identify, characterize and determine the regulation of the genes involved in the biosynthesis of off-flavor metabolites in aquaculture systems.

Need for Research

-Description of Problem to be Solved -

Off-flavors in catfish aquaculture are primarily due to the production of geosmin and 2-methylisoborneol (MIB) by certain cyanobacterial species that inhabit aquaculture systems. Accumulation of these compounds in the flesh renders fish unmarketable and is reported to cost the farmers up to 25% of potential revenues. However, benign (non-off-flavor producing) cyanobacteria and algae are beneficial to the aquaculture system by providing dissolved oxygen to the fish and reducing the need for mechanical aeration. Therefore, the goal of the proposed research is to develop methods to specifically remove off-flavor metabolites and/or off-flavor producing cyanabacteria from aquaculture systems safely and economically.

Cyanophage are viruses that specifically infect cyanobacteria. Within the past decade it has become well established that the world's oceans are teeming with cyanophage that are specific for certain species of cyanobacteria and contribute mightily to the overall ecology of the resident phytoplankton. More recently, it has been revealed that most, if not all fresh-water sources also harbor cyanophage, including catfish aquaculture systems. Research is needed to determine if cyanophage can be employed as a specific biocide for off-flavor-producing cyanobacteria in catfish ponds.

Bacteria that can use geosmin and/or MIB as sole carbon and energy source for growth may be useful for removing geosmin and MIB from catfish ponds. Research is needed to develop strains for prevention or bioremediation of these off-flavor chemicals.

Geosmin and MIB are the two prevalent off-flavor metabolites. They impart a musty/earthy taste and smell when present. The biosynthetic pathway for geosmin and MIB is not established in cyanobacteria or the well-studied *S. coelicolor*, a soil-dwelling, geosmin-producing organism. Research is needed to reveal the genes, encoded-enzymes and regulatory system controlling geosmin and MIB biosynthesis. This information is important for the development of effective methods for inhibiting off-flavor metabolite production.

-Relevance to ARS National Program Action Plan - NP 106 Aquaculture -

This program falls under components of the Aquaculture Action Plan. Of particular relevance is the section entitled “Quality, Safety and Variety of Aquaculture Products.” The proposed research directly addresses subsection d, *Off-flavor Delayed Harvesting* parts 1 and 2, “Discovery and development of natural, selective algicides to manage the most common environmentally-derived, off-flavor compounds,” and “Discovery and development of organisms for the remediation of off-flavors,” respectively, as well as, subsection e *Off-flavor Methodology* parts 2 and 3, “Determine the biological pathways and regulatory steps of common off-flavor compounds,” and “Develop methods for controlling the biological pathways of common off-flavor compounds,” respectively.

-Potential Benefits -

Successful completion of the proposed research will have a dramatic and positive benefit for farmers and the public. In an environment in which off-flavor episodes are controllable or completely eliminated, the farmers will be able to harvest as the market demands, rather than when off-flavors allow. Further, the economic boost or lowering of the costs of production lost to off-flavor episodes should have a positive impact for both the farmers and the consuming public.

-Anticipated Products -

Each of the objectives has the potential to yield novel products for the control of off-flavors. For example, the work described in Objective 1 may reveal biological agents (viruses) that are effective in specifically killing off-flavor-producing species of cyanobacteria. The combination of approaches under Objective 2 will lead to the isolation and characterization of bacteria that degrade geosmin and 2-methylisoborneol. These bacteria will be developed for use in eliminating those off-flavor chemicals from catfish aquaculture.

Whereas, the research described in Objective 3 is the standard approach used towards understanding nearly any biological process from development of cancer in humans to metabolism in bacteria, predicting the actual agent that would be effective at disrupting the biosynthesis of off-flavor compounds would be difficult at best. Nevertheless, it is expected that some small molecule effector(s) would be identified in the future based on this approach that would suit the purposes of the farmer.

-Customers -

Farmers, particularly those of catfish, and catfish processors would be the primary customers.

Scientific Background

This project is coordinated with CRIS 6408-41000-003-00D at University of Mississippi, Oxford, MS entitled “Control of Undesirable Microbes and Off-flavors in Aquaculture.” In that project, natural products will be screened in the laboratory and successful candidates further tested for efficacy. Thus, these projects are complementary and non-overlapping. This project is also coordinated with CRIS 6402-13320-002-00D at the Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS, entitled “Optimizing Catfish/water Quality

Interactions to Increase Catfish Production Efficiency." We have a long-standing collaboration with Dr. Paul Zimba. These projects are also complementary and non-overlapping.

Approach and Research Procedures

Presented below are three independent objectives that serve as contingencies for each other and will be pursued concurrently. Objectives 1 and 2 are particularly attractive because they are realistic and relatively short-term solutions to the problems of off-flavors in aquaculture. Should research progress towards either of these objectives show extreme promise, the focus of the group's effort would shift towards that objective. The work described under Objective 3 seeks to build an understanding of off-flavor metabolite biosynthesis that would be sufficient to devise strategies to inhibit the pathway. Such inhibitors would be developed in the future. Whereas, two approaches are presented for this objective, a third is presented as a contingency.

Objective 1:

Identify, characterize and determine the killing efficiency of viruses specific for off-flavor producing cyanobacteria in aquaculture systems.

Hypothesis -

Cyanophage can be discovered and used as a specific biocide for off-flavor-producing cyanobacteria.

Experimental design -

PCR-based assays will be used to characterize the abundance and variety of cyanophage present in aquaculture systems at various times during the year. These data will be compared to the cyanobacteria present. The morphology of the cyanophage present will be examined by transmission electron microscopy. Briefly, degenerate primers will be used to amplify conserved regions of the cyanophage genome. The unique DNA fragments will be separated by denaturing gradient gel electrophoresis and cloned. The phage will be shown to kill relevant (geosmin- or MIB-producing) cyanobacteria. The species-specificity of the phage will be determined by plaque assays with cultured cyanobacteria. The entire genome of phage shown to be specific for off-flavor-producing species will be determined. The latter information will facilitate understanding the phage's life cycle and aid in development of it as a biocidal agent for control of off-flavors.

Collaborations -

Internal to ARS: Dr. Paul Zimba, Stoneville, MS. External to ARS: Dr. Curtis Suttle, University of British Columbia.

Objective 2:

Identify, characterize and develop novel bacterial strains that metabolize (biodegrade) geosmin and 2-methylisoborneol and develop them for use in eliminating those chemicals from aquaculture systems.

Hypothesis -

Bacteria can be utilized to remove off-flavor metabolites from aquaculture systems.

Experimental design -

Using selective enrichment, bacteria that can use geosmin or MIB as sole carbon and energy source for growth have been, and will continue to be, isolated from various environments. Some of these bacteria may be useful for removing geosmin and MIB from catfish ponds. To develop strains for prevention or bioremediation of these off-flavor chemicals, several lines of research will be followed. Studies of the kinetics of geosmin and MIB metabolism will identify those bacterial strains that degrade geosmin and MIB at adequate rates and at relevant substrate concentrations for use in aquaculture treatment. Odor thresholds for humans are about 10 ng/L (55 pM) for geosmin and 30 ng/L (180 pM) for MIB, therefore it will be necessary for bacteria to degrade geosmin and MIB at these very low concentrations. Routine assays involving the identification and quantitation of geosmin, MIB and their metabolites extracted from culture supernatants will be carried out using gas chromatography-mass spectrometry (GC-MS). The pathways by which selected bacteria convert geosmin and MIB to small molecules of central metabolism will be studied using a combination of biochemical and genetic approaches. These may include isolation of blocked mutants following transposon or chemical mutagenesis, cloning of genes encoding enzymes that catalyze reactions of the pathway, purification of certain pathway enzyme(s). The resulting bacteria or enzymes will be used to produce and accumulate pathway intermediates which can be isolated and identified. Bacterial catabolic pathways are often tightly regulated and inducible by a pathway substrate or intermediate; it is reasonable to expect that geosmin and MIB are the inducers of the enzymes that degrade them. To employ bacteria in removing geosmin and MIB from catfish ponds, it would be useful to be able to produce sufficient quantities of bacteria that are actively producing enzymes that metabolize geosmin and MIB without adding those chemicals. It is, therefore, necessary to determine the normal inducers and potential alternative inducers of the pathway, and if possible, to isolate unregulated (constitutive) mutant strains that do not require inducer chemicals. Bacterial strains will be identified to genus and species by sequencing DNA encoding 16S ribosomal RNAs. This will prevent the use of potentially pathogenic strains and also may suggest genetic approaches for studying metabolic pathways and for strain improvement.

Collaborations -

Internal to ARS: Dr. Casey Grimm, SRRC-FPSQ.

Objective 3:

Identify, characterize and determine the regulation of the genes involved in the biosynthesis of off-flavor metabolites in aquaculture systems.

Hypothesis -

Small molecules that interfere with the normal regulation of the biosynthetic pathways for off-flavor metabolites can be developed.

Experimental design -

Two approaches will be utilized. The gene for a sesquiterpene cyclase shown to be critical for the synthesis of geosmin in *S. coelicolor* has been reported. The DNA sequence of this

gene will be used to design degenerate primers for PCR amplification and cloning of homologous genes in relevant cyanobacteria. Those genes will be sequenced, as well as that of the flanking DNA regions. Since metabolically-related genes are often clustered on the chromosomes of bacteria, other genes involved in geosmin biosynthesis may be identified. While a reasonable sequence of reactions leading from the cyclization product to geosmin has been proposed based on experiments involving incorporation of labeled precursors by geosmin-producing *Streptomyces* sp. and *Fossombronia pusilla* (liverwort), the individual reactions, the enzymes that catalyze the reactions, and the genes that encode the enzymes have not been studied.

In the second approach, transposon mutagenesis will be used to disrupt all the genes involved in either geosmin or MIB synthesis, as well as their regulators. Transposon mutants will be screened for the lack of geosmin or MIB synthesis with monoclonal antibodies in ELISA format. The disrupted genes will be revealed by inverse PCR, cloning and subsequent sequence analysis. Confirmation of a particular gene's involvement in the biosynthetic pathway will be done by specifically knocking out the wild type gene in a previously unmutagenized strain.

Contingency -

If the two approaches described above are not successful, the standard, but highly-laborious biochemical strategy will be pursued. Briefly, enzyme assays will be developed for supposed steps in the biosynthetic pathways. The enzyme responsible for the activity will be purified from crude lysates of off-flavor producing organisms. N-terminal and internal peptide fragments will be sequenced and degenerate oligonucleotides will be designed based on those sequences. These will be used in either PCR or blotting experiments to isolate a DNA fragment encoding part of the enzyme. The intact gene will then be identified by further blotting experiments. This is the strategy that Dr. Hu at Arizona State University is supposed to pursue in the coming years.

Collaborations -

Internal to ARS: Dr. Casey Grimm, SRRC-FPSQ, Dr. Paul Zimba, Stoneville, MS. External to ARS: Dr. Qiang Hu, Arizona State University.

Conflicts of Interest

See attached lists.